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Vascular endothelial growth factor correlates with matrix metalloproteinase-9 in the pleural effusion

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Summary Vascular endothelial growth factor (VEGF) is a potent, multifunctional cytokine that contributes to angiogenesis and inflammation. Matrix metalloproteinase-9 (MMP-9) is one of the major proteolytic enzymes that degrade various components of the extracellular matrix. Few data are available on the potential relationship between VEGF and MMP-9 in the accumulation of pleural effusion. We examined levels of VEGF and MMP-9 by means of enzyme immunoassay, zymographic analysis, and Western blot analysis in the patients with liver cirrhosis, tuberculosis, or lung cancer. The levels of VEGF and MMP-9 were significantly increased in the pleural fluids and sera of patients with tuberculosis and were even higher in patients with lung cancer compared with the patients with liver cirrhosis. A significant correlation was established between the level of VEGF and the level of MMP-9 in the pleural effusion. These results suggest that overproduction of VEGF and MMP-9 is associated with accumulation of the pleural effusion in tuberculosis and lung cancer. The relationship between VEGF and MMP-9 in the pleural effusion may have a role in the pathogenesis of pleural fluid formation.

Introduction

Pleural effusion is seen in patients with various benign and malignant diseases such as liver cirrhosis, tuberculosis, or lung cancer. However, the basic mechanism by which fluid accumulates within the pleural space is poorly understood. Vascular endothelial growth factor (VEGF) is one of the most potent proangiogenic cytokines and exhibits mitotic activity specific for vascular endothelial cells. VEGF also enhances a vascular permeablilty and plays a critical role in tumor progression probably due to its angiogenesis action.[1,2]

Matrix metalloproteinases (MMPs) are proteolytic enzymes involving in turnover of extracellular matrix (ECM) proteins. These enzymes are known to play an important role in pathologic conditions such as tumor invasion.[3,4] Of the MMP family, MMP-9 may play a role in chronic inflammation. The enzyme induces migration of inflammatory cells such as eosinophils, neutrophils, and lymphocytes across basement membrane. Furthermore, there is evidence that VEGF stimulates MMP-9 secretion.[5] However, few data are available on the potential relationship between VEGF and MMP-9 in the accumulation of pleural effusion.

In the present study, we evaluated whether the VEGF and MMP-9 participate in the pathogenesis of pleural fluid formation of patients with liver cirrhosis, tuberculosis, or lung cancer. An additional aim of the present study was to determine whether

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the level of VEGF in the pleural fluids of patients with liver cirrhosis, tuberculosis, or lung cancer correlated with the level of MMP-9.

Material and methods

Subjects

Fourty patients with lung cancer, 27 patients with tuberculosis, and 16 patients with liver cirrhosis were recruited from the Chonbuk National University Hospital. The pleural effusion was classified as exudates or transudates according to the Light's criteria.[6]

Exudate ($n = 67$): when the ratio of pleural fluid/serum proteins is higher than 0.5, pleural fluid/serum LDH > 0.6, or pleural fluid LDH $> 2/3$ of the upper normal limit in serum LDH.

- *Lung cancer* ($n = 40$): Diagnosis was established by pleural fluid cytology in 12 patients and histologic finding on a pleural biopsy specimen in 28 patients (19 with squamous cell carcinoma, nine with small cell carcinoma, 12 with adenocarcinoma).
- *Tuberculosis* ($n = 27$): Diagnosis was established by sputum AFB stain or positive culture in 10 patients and typical histological characteristics on a tissue biopsy specimen in 17 patients.

Transudate; liver cirrhosis ($n = 16$): when the ratio of pleural fluid/serum proteins is lower than 0.5, pleural fluid/serum LDH < 0.6, and pleural fluid LDH $< 2/3$ of the upper normal limit in serum LDH. Diagnosis was established when thrombocytopenia and the reverse of albumin/globulin ratio in laboratory findings, splenomegaly or hepatic shirinkage including irregularity of margin and ascite formation in ultrasonography, and esophageal varix in upper esophagogastric endoscopy were present.

This study was approved by the Ethics Committee of Chonbuk National University, Medical School and fully informed, written consent was obtained from each subject.

Sample processing

Pleural effusion was collected via diagnostic thoracentesis. After centrifugation at 3000 rotations/min for 20 min at 4°C, the cell-free supernatant was separated and stored at −70°C for subsequent VEGF and MMP-9 measurement. Serum samples were simultaneously collected and stored at −70°C.

Cellular and biochemical analysis of pleural effusion

At the time of the thoracentesis, pleural fluid was collected in an ethylenediamine tetraacetic acid tube for the measurement of the total and differential WBC count, and in a plain tube for glucose, protein, LDH, and adenosine deaminase (ADA) analysis. WBC count was obtained by manually counting 100 cells on a smear stained with Wright's stain after the cells had been concentrated by cytocentrifugation at 2000 rotations/min for 10 min. Glucose, protein, and LDH concentrations were measured using as automated analyzer (Vitro Model 950; Johnson & Johnson: New York, NY, USA). ADA activity was measured with a commercial assay kit (Toyobo Co., Osaka, Japan).

Enzyme linked immunosorbent assay of VEGF and MMP-9

The levels of VEGF were determined using a human VEGF enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA). The levels of MMP-9 were quantified by enzyme immunoassays according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan). The minimum detectable levels of VEGF and MMP-9 are 5.0 pg/ml and 1.2 ng/ml, respectively.

Gelatin zymography of MMP-9

The hydrolytic activity of MMP-9 in pleural effusion was measured by gelatin zymography. Zymography was performed as previously desecribed.[7] Briefly, samples containing the same amount of protein (10 μg) were mixed with 5 × sample buffer (0.4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and were separated by 10% SDS-PAGE gels containing 0.1% gelatin. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h and were then placed in the enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) for 24 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue-250 solution and destained with several changes of 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as clear bands against a blue background.

Western blot analysis of VEGF

Each pleural fluid supernatant was quantified using the Bradford reagent (Bio-rad) and 3 μg of pleural fluid protein was loaded on 12% SDS-PAGE gel and

separated at 120 V for 90 min. After electroporesis, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% nonfat milk in TBST buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 2 h and the anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, was then incubated for 2 h in TBST buffer at room temperature. Specific binding was visualized using enhanced chemiluminescence system reagents (Amersham Pharmacia), and exposed to photographic film.

Statistical analysis

All values are reported as means $\pm$ SD unless otherwise stated. The Kruskal–Wallis analysis of variance test was used to examine significant intergroup differences and if significant, the Mann–Whitney *U* test was used for between-group comparisons. Spearman's rank correlation was calculated to assess the correlation between data. Data were considered statistically significant if *p* values were less than 0.05. Statistical analysis was performed using the SPSS 8.0 software (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of subjects and pleural effusion

The mean ages of patients with lung cancer (58.3 ± 7.03), tuberculosis (49.1 ± 8.57), and liver cirrhosis (54.8 ± 8.94) were comparable with each other. The numbers of patients and sex ratio of lung cancer ($n = 40$, M/F = 24/16), tuberculosis ($n = 27$, M/F = 14/13), and liver cirrhosis ($n = 16$, M/F = 10/6) were comparable with each other (Table 1). Total WBC numbers and LDH levels in the pleural fluids were significantly elevated in the patients with lung cancer and tuberculosis as compared to the patients with liver cirrhosis. However, no significant difference in these levels was observed between the lung cancer and the tuberculosis patients. ADA levels in the pleural fluids were significantly higher in the patients with tuberculosis than the patients with lung cancer and liver cirrhosis. The numbers of lymphocytes in the pleural fluids of the patients with lung cancer and tuberculosis were significantly increased as compared to the patients with liver cirrhosis. However, the numbers of lymphocyte were similar to the lung cancer and the tuberculosis patients (Table 2). The levels of VEGF in the pleural fluids were increased approximately 14.5 fold in the patients with tuberculosis and approximately 34.2 fold in the patients with lung cancer as compared to those of liver cirrhosis patients. The levels of MMP-9 in the pleural fluids were also increased approximately 3.2 fold in the patients with tuberculosis and approximately 6.9 fold in the patients with lung cancer as compared to those of liver cirrhosis patients (Table 2).

VEGF levels in pleural effusion

Enzyme immunoassay showed that the concentration of VEGF normalized to pleural fluid protein content was significantly increased in the patients with tuberculosis and were even higher in the patients with lung cancer than those of the patients with liver cirrhosis. The levels of VEGF in the pleural fluids of patients with lung cancer were also significantly elevated as compared to the patients with tuberculosis (Fig. 1A). However, there was no significant difference in VEGF levels in the pleural fluids of patients with lung cancer of different histological types. Consistent with the results obtained from enzyme immunoassay, Western blot analysis revealed that VEGF levels were increased in the pleural fluids of patients with tuberculosis and were even higher in the patients with lung cancer compared with the patients with liver cirrhosis. And, the levels of VEGF in the pleural fluids of patients with lung cancer were higher than the patients with tuberculosis (Fig. 1B).

Table 1 Patients characteristics.

	Transudate	Exudate	
Disease	Liver cirrhosis	Tuberculosis	Lung cancer
Number	16	27	40
Age (yr)	54.8 ± 8.94	49.1 ± 8.57	58.3 ± 7.03
Sex (M/F)	10/6	14/13	24/16

Data are presented as means $\pm$ SD.

Table 2 Characteistics of pleural effusions in different diseases.

	Transudate	Exudate	
Disease	Liver cirrhosis	Tuberculosis	Lung cancer
WBC (cells/μl)	263.2±26	2437.1±1527.1*	2066.5±681.8*
Lymphocyte (cells/μl)	228.8±21.1	2217.8±170.6*	1859.0±165.3*
Protein (g/dl)	1.3±0.7	3.9±0.6*	3.7±1.2*
Glucose (mg/dl)	95.2±23.9	98.1±35.0	107.5±35.0
LDH (IU/l)	121.1±39.1	650.16±273.1*	422.6±177.7*
ADA (IU/l)	6.5±3.1	72.8±28.7*†	7.6±5.1
VEGF (pg/ml)	75.8±77.3	1100.5±368.4*†	2595.7±718.5*
MMP-9 (ng/ml)	33.0±16.0	1.06.3±30.1*†	226.6±64.4*

*$P<0.01$ versus liver cirrhosis; †$P<0.01$ versus lung cancer.
LDH = lactate dehydrigenase(normal; <200 IU/l).
ADA = adenosine deaminase.
Data are presented as means±SD.

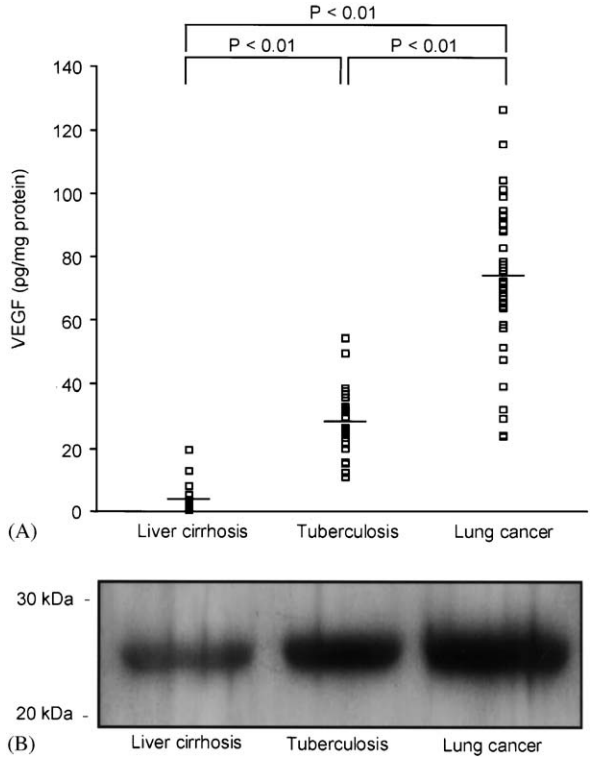

Figure 1 (A) The levels of VEGF in the pleural fluids of patients of liver cirrhosis, tuberculosis, or lung cancer by enzyme immunoassay; and (B) Western blot analysis of VEGF in the pleural effusions of liver cirrhosis, tuberculosis, or lung cancer.

MMP-9 levels in pleural effusion

Enzyme immunoassay showed that the concentration of MMP-9 normalized to pleural fluid protein content was significantly elevated in the pleural fluids of patients with tuberculosis and were even higher in the patients with lung cancer than those of the patients with liver cirrhosis. The levels of MMP-9 in the pleural fluids of the patients with lung cancer were also significantly increased as compared to those of the patients with tuberculosis (Fig. 2A). However, there was no significant difference in MMP-9 levels in the pleural fluids of patients with lung cancer of different histological types. Consistent with the results obtained from enzyme immunoassay, gelatin zymography revealed that the levels of MMP-9 were also increased in the pleural fluids of the patients with tuberculosis and were even higher in the patients with lung cancer than the patients with liver cirrhosis. The levels of MMP-9 in the pleural fluids of patients with lung cancer were higher than the patients with tuberculosis (Fig. 2B).

The correlation of VEGF with MMP-9 in pleural effusion

In the pleural fluids of the patients with liver cirrhosis, tuberculosis, or lung cancer, levels of VEGF were significantly correlated with those of MMP-9 ($r = 0.883; P<0.01$) (Fig. 3).

The correlation of lymphocytes with the levels of VEGF or MMP-9

In the pleural fluids of the patients with liver cirrhosis, tuberculosis, and lung cancer, levels of VEGF or MMP-9 were significantly correlated with the number of lymphocytes ($r = 0.556; P<0.01$ or $r = 0.511; P<0.01$, respectively) (Fig. 4).

Serum levels of VEGF and MMP-9

Enzyme immunoassay revealed that the levels of vegf and mmp-9 were increased in the sera of

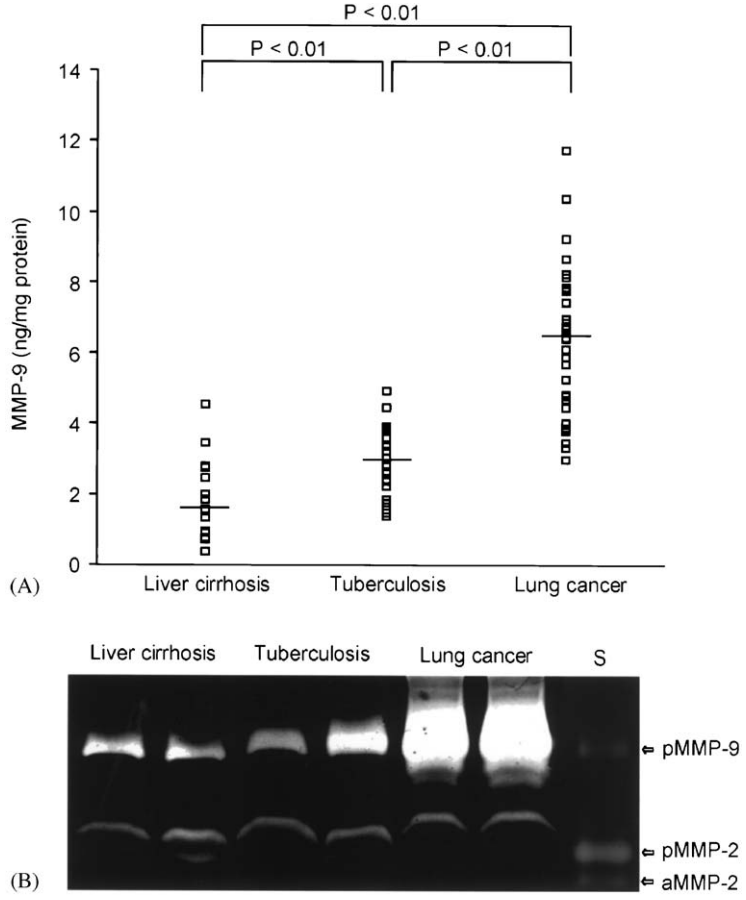

Figure 2 (A) The levels of MMP-9 in the pleural fluids of patients with liver cirrhosis, tuberculosis, or lung cancer by enzyme immunoassay; and (B) gelatin zymography of pleural effusions of liver cirrhosis, tuberculosis, or lung cancer. A representative zymographic analysis of pleural fluid supernatant samples obtained from liver cirrhosis (lanes 1,2), tuberculosis (lanes 3,4), and lung cancer (lanes 5,6). Lanes S contains standards, the pro-form of MMP-2 (pMMP-2), the active form of MMP-2 (aMMP-2), and the pro-form of MMP-9 (pMMP-9).

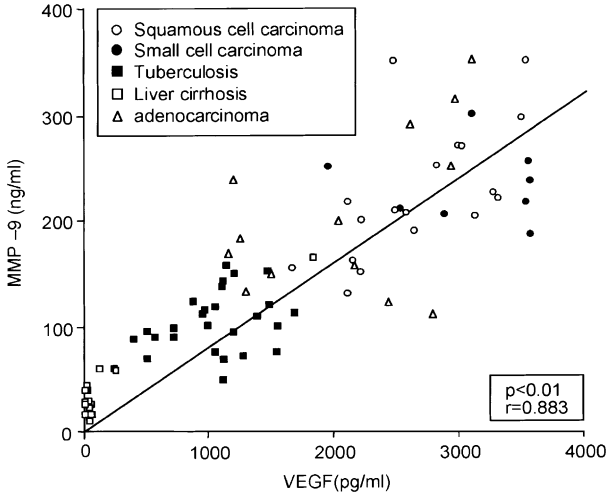

Figure 3 The correlation of the levels of VEGF with the levels of MMP-9 in the pleural fluids of patients with liver cirrhosis, tuberculosis, or lung cancer.

patients with tuberculosis (989.4 $\pm$ 491.1 pg/ml and 397.6 $\pm$ 204.3 ng/ml) and were even higher in the patients with lung cancer (1638.7 $\pm$ 617.1 pg/ml and 949.8 $\pm$ 391.0 ng/ml) than those of the patients with liver cirrhosis (413.4 $\pm$ 254.1 pg/ml and 97.6 $\pm$ 52.6 ng/ml). the levels of vegf and mmp-9 in the sera of patients with lung cancer were significantly higher than the patients with tuberculosis (Fig. 5).

Discussion

Abnormal accumulation of pleural fluid probably occurs due to a combination of increased pleural fluid formation and in some cases, decreased lymphatic drainage.[8,9] Increased permeability of the capillaries in visceral pleura is considered to

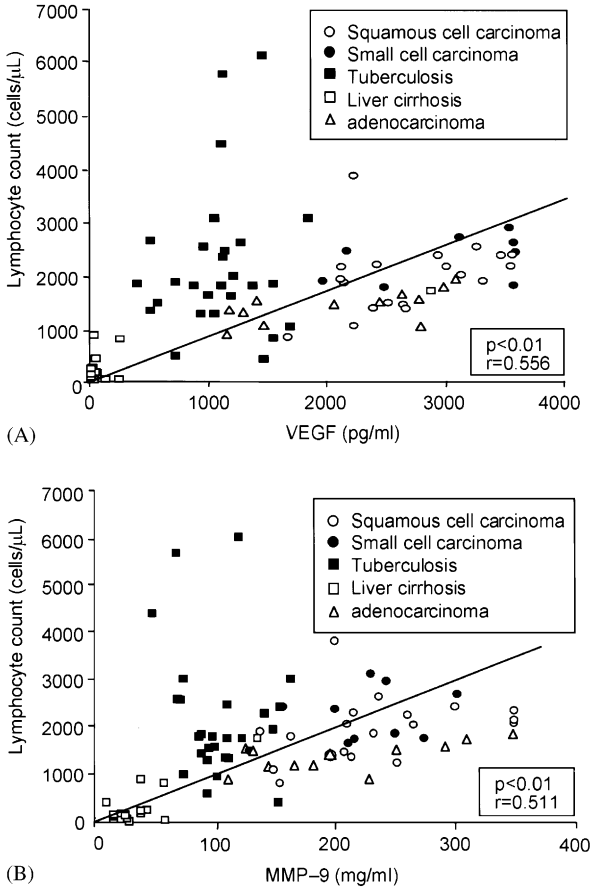

Figure 4 (A) The correlation of the number of lymphocytes and the levels of VEGF in the pleural effusions of liver cirrhosis, tuberculosis or lung cancer; and (B) the correlation of the number of lymphocytes with the levels of MMP-9 in the pleural fluids of patients of liver cirrhosis, tuberculosis, or lung cancer.

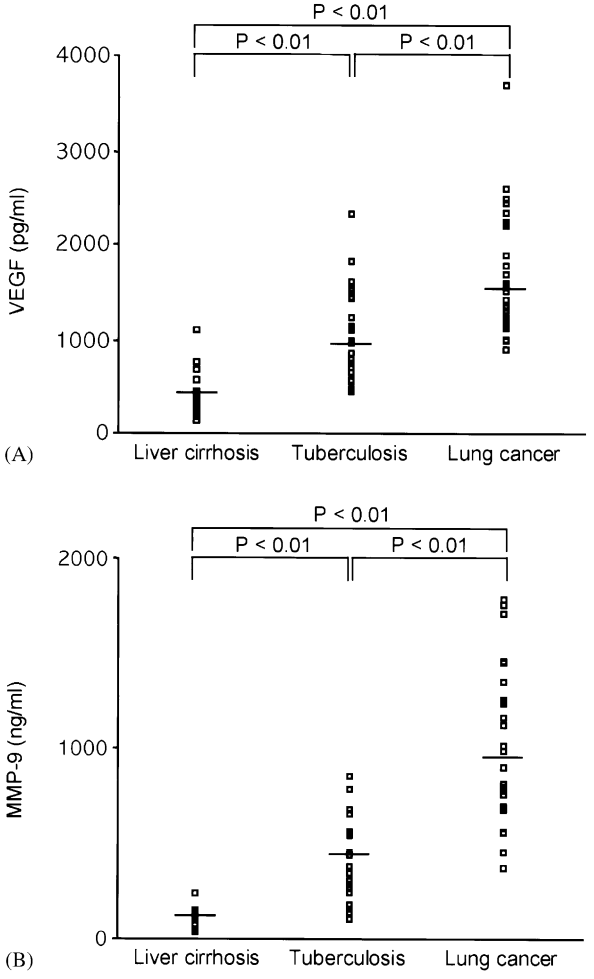

Figure 5 The levels of VEGF (A); and MMP-9 (B) in the sera of patients of liver cirrhosis, tuberculosis or lung cancer by enzyme immunoassay.

play an important role in the production of exudative pleural effusion.[9,10] However, the molecular mechanisms or pathogenesis in pleural fluid accumulation are poorly understood and no single mediator has so far been found to be important in all types of exudative pleural effusion. Especially, the origin of pleural effusion in malignancy is not definitely known and the pathogenesis is probably multifactorial.[11]

In our study, the levels of VEGF are high in the pleural fluids of patients with tuberculosis and even higher in the patients with lung cancer compared with the patients with liver cirrhosis. We also showed that the levels of VEGF were significantly correlated with the number of lymphocytes in the pleural fluids of patients with liver cirrhosis, tuberculosis, and lung cancer. Increased pleural permeability and local interaction of cells and cytokines are associated with the pleural effusion of tuberculosis caused by reactive response of lymphocytes.[12] VEGF is produced by most inflammatory cells including eosinophils, neutrophils, macrophages, and lymphocytes.[13] VEGF produced by lymphocytes may be present in the pleural fluids of patients with tuberculosis. VEGF is an endothelial cell-specific potent, multifunctional cytokine that plays an important role in vasculogenesis and angiogenesis. Interestingly, formation of VEGF mRNA is found in almost all tumors.[1,14–16] The contribution of VEGF to pleural fluid accumulation through increment in vascular permeability has also been reported in an animal model.[17] Its ability to increase vascular permeability and contribution to tumor angiogenesis at the site of tumor progression has been also observed.[18] Yeo et al.[19] showed that significant amount of VEGF was present in malignant pleural effusion associated with lung cancer, and the VEGF present in the pleural fluid was produced by the malignant cells as well as monocytes, macrophages, or lymphocytes in the pleural

fluids.[10] In addition, it is possible that VEGF from the tumor tissue enter the pleural space and could increase the permeability of capillaries in the pleura, leading to increase pleural fluid formation.[15] Accordingly, VEGF can be detected in the malignant pleural effusion with lung cancer. Taken together, we suggest that the sources of VEGF may be malignant cells as well as inflammatory cells including lymphocytes and that VEGF may play a crucial role in the pathogenesis of pleural effusion of tuberculosis and lung cancer.

In our study, the levels of MMP-9 are high in the pleural fluids of patients with tuberculosis and even higher in the patients with lung cancer compared with the patients with liver cirrhosis. The pleural effusion of tuberculosis caused by reactive response of lymphocytes is associated with increased pleural permeability and local interaction of cells and cytokines, which possibly contribute to MMP-9 accumulation.[12] In this study, we showed the correlation between the number of lymphocytes and the levels of MMP-9 in the pleural fluids of patients with liver cirrhosis, tuberculosis, and lung cancer. MMP-9 produced by lymphocytes may be present in the pleural fluids of patients with tuberculosis. Inflammatory cells can produce MMP-9, which might contribute to ECM degradation and absorption in numerous pathophysiological processes including infection and immunologically mediated diseases.[20,21] In addition to fibroblast, endothelial cells, granulocyte, and macrophage, tumor cells may also contribute to the production of MMP-9 Proteolysis of basement membrane gelatin/type-IV collagen by MMP-9 is considered to be a crucial component of cancer invasion and metastasis. Therefore, MMP-9 can be detected in the malignant pleural effusion. This contributes to formation of pleural effusion of inflammatory or malignant diseases. Taken together, we suggest that malignant cells may be partly involved in the production of MMP-9 in addition to inflammatory cells such as lymphocytes, and MMP-9 may play a crucial role in the pathogenesis of pleural effusion of tuberculosis or lung cancer.

In our study, a significant correlation was found between the levels of VEGF and the levels of MMP-9 in the pleural fluids of patients with liver cirrhosis, tuberculosis, or lung cancer. It is possible that inflammatory cells and tumor cells are sources of VEGF and MMP-9 and are involved in the formation of pleural fluid. Previously, Wang et al.[5] also showed that VEGF enhances MMP-9 secretion from human smooth muscle cells. Furthermore, the presence of relatively high concentration of these proteins in the pleural fluids of patients with lung cancer suggests that they might serve as important function in accumulation of pleural fluid of patients with lung cancer.

Conclusively, we believe that, at least, VEGF and MMP-9 are involved in the pathogenesis of pleural effusion of patients with tuberculosis or lung cancer in view of the increment and correlation between them, especially malignant pleural effusion caused by lung cancer. In addition to inflammatory cells such as lymphocytes, tumor cells may be partly involved in the production of VEGF and MMP-9. The clinical use of VEGF and MMP-9 measurement in the pleural effusion as a differential diagnostic parameter or diagnostic reference may be possible with other parameters. However, further studies are needed to determine the clinical value of soluble VEGF and MMP-9 in diagnostic and therapeutic aspects in pleural effusion.

Acknowledgements

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